Glycosylated Fibronectin as a First-Trimester Biomarker for Prediction of Gestational Diabetes

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OBJECTIVE: To evaluate the potential clinical utility of serum biomarkers for first-trimester prediction of gestational diabetes mellitus (GDM).

METHODS: Maternal serum concentrations of glycosylated (*Sambucus nigra* lectin–reactive) fibronectin, adiponectin, sex hormone–binding globulin, placental lactogen, and high-sensitivity C-reactive protein (CRP) were measured at 5–13 weeks of gestation in a case-control study of 90 pregnant women with subsequent development of GDM and in 92 control group participants. Ability to detect GDM was assessed using logistic regression modeling and receiver operating characteristic (ROC) curves. Classification performance and positive and negative predictive values were reported at specific thresholds. Glycosylated fibronectin variation across trimesters was evaluated using a serial-measures analysis of 35 nondiabetic control group participants.

RESULTS: First-trimester serum concentrations of glycosylated fibronectin, adiponectin, high-sensitivity CRP, and placental lactogen were significantly associated ($P<.001$) with GDM. After adjustment for maternal factors and other biomarkers, glycosylated fibronectin demonstrated an independent association with GDM ($P<.001$). Adiponectin, high-sensitivity CRP, and placental lactogen demonstrated modest classification performance compared with glycosylated fibronectin (respectively: area under the curve [AUC] 0.63; 95% confidence interval [CI] 0.53–0.71; AUC 0.68; 95% CI 0.60–0.76; and AUC 0.67, 95% CI 0.59–0.75; compared with AUC 0.91; 95% CI 0.87–0.96). Glycosylated fibronectin levels above a threshold of 120 mg/L correctly identified 57 GDM case group participants with a positive predictive value of 63% (95% CI 53–72%) and a negative predictive value of 95% (95% CI 94–95%) at a population prevalence of 12%. There was no association between sex hormone–binding globulin and GDM.

CONCLUSION: First-trimester glycosylated fibronectin is a potential pregnancy-specific biomarker for early identification of women at risk for GDM.

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LEVEL OF EVIDENCE: II

Standard assessments of diabetes and prediabetes, such as fasting insulin and glucose and glycated hemoglobin A_{1c}, are not recommended for gestational diabetes mellitus (GDM) screening. The standard screening modality in the United States is currently a 50-g, 1-hour oral glucose tolerance test (OGTT) performed at 24–28 weeks of gestation;[1] however, the pooled sensitivity and specificity of this test are only 74% (95% confidence interval [CI] 62–87%) and 77% (95% CI 66–89%), respectively.[2] New diagnostic approaches that allow earlier assessment can facilitate a shift from current standard-of-care practices by enabling earlier treatment. The desirability of early detection and treatment is

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generally accepted on a theoretical basis,[3] but the availability of a robust early screening test will allow the evaluation of the efficacy of early treatment in reducing gestational and postgestational risks for mother and newborn.

Proteins modified by intracellular glycosylation as opposed to nonenzymatic glycation of factors such as glycated hemoglobin A_{1c} or albumin could be better indicators of maternal response to metabolic shifts in pregnancy, and altered secretion of glycoproteins has been described in GDM.[4] Based on our previous proteomic analysis of maternal serum that demonstrated alterations in the glycoproteome in gestational complications,[5] we evaluated glycosylated (*Sambucus nigra* lectin–reactive) fibronectin to ascertain its potential utility as an early GDM biomarker, and we found in preliminary studies that glycosylated fibronectin was significantly elevated in first-trimester GDM maternal serum compared with nondiabetic maternal serum. In the current study, glycosylated fibronectin levels were evaluated in a larger study population, and its performance compared with other potential GDM biomarkers, including adiponectin,[6–9] sex hormone–binding globulin,[7,10–12] high-sensitivity C-reactive protein (CRP),[13,14] and placental lactogen.[15,16]

MATERIALS AND METHODS

Study participants were selected from the Finnish maternity serum bank at the National Institute for Health and Welfare. Recruitment occurred between 2004 and 2010 from maternity clinics in the area of Oulu University Hospital (Oulu, Finland) and Kuopio University Hospital (Kuopio, Finland). Blood samples were taken from women participating in a first-trimester screening program for trisomies. All clinical data were obtained from the Birth Register, a computerized database containing information about baseline maternal characteristics and pregnancy outcome. The study was approved by the Ethics Committees at Oulu University Hospital and Kuopio University Hospital. All participants provided informed consent.

The current study used a case-control design in which GDM case group participants and nondiabetic control group participants were retrospectively selected from the described population (Fig. 1). Participants needed a sufficient quantity of first-trimester serum collected between 5 weeks and 13 weeks of gestation and had to complete a 75-g, 2-hour OGTT for inclusion. Eligible GDM case group participants included any woman with development of GDM during pregnancy, identified by OGTT. A diagnosis of GDM was made if fasting, 1-hour, or 2-hour glucose values exceeded upper normal limits (95 mg/dL [5.3 nmol/L], 180 mg/dL [10.0 nmol/L], and 155 mg/dL [8.6 nmol/L], respectively). Using the same diagnostic criteria, nondiabetic control group participants had normal glucose tolerance and did not have development of GDM during pregnancy. Exclusion criteria were multiple gestations and pregnancy complications, including preeclampsia or preterm labor. There were no ethnic differences between the groups.

Sample size was determined from the degree of separation between GDM case and control group participants observed in a pilot study (n=29; unpublished data). Based on the observed performance characteristics of the glycosylated fibronectin assay in this study and minimum expected true-positive and false-positive fractions of 0.65 and 0.25, respectively, a sample size of 100 GDM case group participants and 100 control group participants exhibited 84% power using calculations described by Pepe.[17] Of the original 200 participants, 15 were not eligible because of insufficient volume of serum for analysis (n=13) or pre-existing diabetes (n=2). Two participants were excluded because of a diagnosis of GDM within 2 weeks of sample collection and one participant was excluded because of missing information about the date of sample collection. The remaining 182 participants (90 GDM case group participants and 92 control group participants) comprised the population described in this study.

Maternal blood was spun, aliquoted, and stored at −80°C according to maternal serum bank protocol. The OGTT plasma glucose was determined using a Konelab 60i Clinical Chemistry Analyzer. Levels of protein analytes were determined by sandwich enzyme-linked immunoassay using specific monoclonal and polyclonal antibodies. Primary antibodies used in enzyme-linked immunoassays included the following: sex hormone–binding globulin monoclonal antibody (31401); placental lactogen monoclonal antibody (L1022-03 G); fibronectin monoclonal antibody (MAB1918); CRP polyclonal antibody (842676); and adiponectin monoclonal antibody (840965). Primary coating antibodies were resuspended in carbonate buffer (pH 9.6), with 100 mL added to each well of a 96-well Reactibind plate and incubated at 4°C overnight. Plates were blocked with 3% bovine serum albumin in phosphate-buffered saline (pH 7.2). After sample addition and incubation for 45 minutes at room temperature, plates were washed with phosphate-buffered saline–Tris buffer using a Biotek plate washer and then incubated with detection antibodies for 45 minutes at room temperature. Plates were again washed with phosphate-buffered saline–Tris buffer and then incubated with streptavidin-horseradish peroxidase (50 ng/mL in phosphate-buffered saline) for 45 minutes

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at room temperature, and then were washed with phosphate-buffered saline–Tris buffer. Tetramethylbenzidine substrate was added and, after the development of the signal, quenched by the addition of 2 N H_2SO_4. Plates were read using an Epoch plate reader at 490 nm, and data were processed using Gen5 software version 1.10.8 and analyzed as described.

For assay of glycosylated fibronectin, the fibronectin monoclonal antibody was used to coat Reactibind plates, which were then blocked as described. Blocking solution was removed, 200 mL per well of oxidation buffer (100 mmol/L sodium periodate and 50 mmol/L citric acid, pH 4.0) was added, and the plate was incubated for 14 minutes. Oxidation solution was removed and the plate was washed with phosphate-buffered saline–Tris buffer. Samples were then applied at a 1:800 dilution, after which the plate was washed with phosphate-buffered saline–Tris buffer. Biotin-conjugated *Sambucus nigra* lectin (Vector Labs) then was added to a final concentration of 0.5 ng/mL in phosphate-buffered saline. After washing the plate with phosphate-buffered saline–Tris buffer, the plate was incubated with streptavidin-horseradish peroxidase and processed as described.

Distributions of maternal characteristics according to study group were compared using two-sided parametric and Wilcoxon nonparametric *t* tests for continuous variables and χ^2 tests for categorical variables. For the serial-measures analysis of glycosylated fibronectin, least square means and 95% CIs by trimester were computed and compared using mixed models. The independent association between each serum biomarker and development of GDM was evaluated using univariate and multivariate logistic regression. Measures of association were reported as unadjusted and adjusted odds ratios (ORs). The first multivariate model included adjustment for maternal age, nulliparity, and gestational age at sample collection. The second model included the same maternal factors in addition to each serum biomarker significantly associated with GDM status ($P<.05$). Glycosylated fibronectin, adiponectin, high-sensitivity CRP, and placental lactogen were included in the full multivariate model.

To evaluate the clinical utility of these analytes as individual biomarkers and as a potential first-trimester

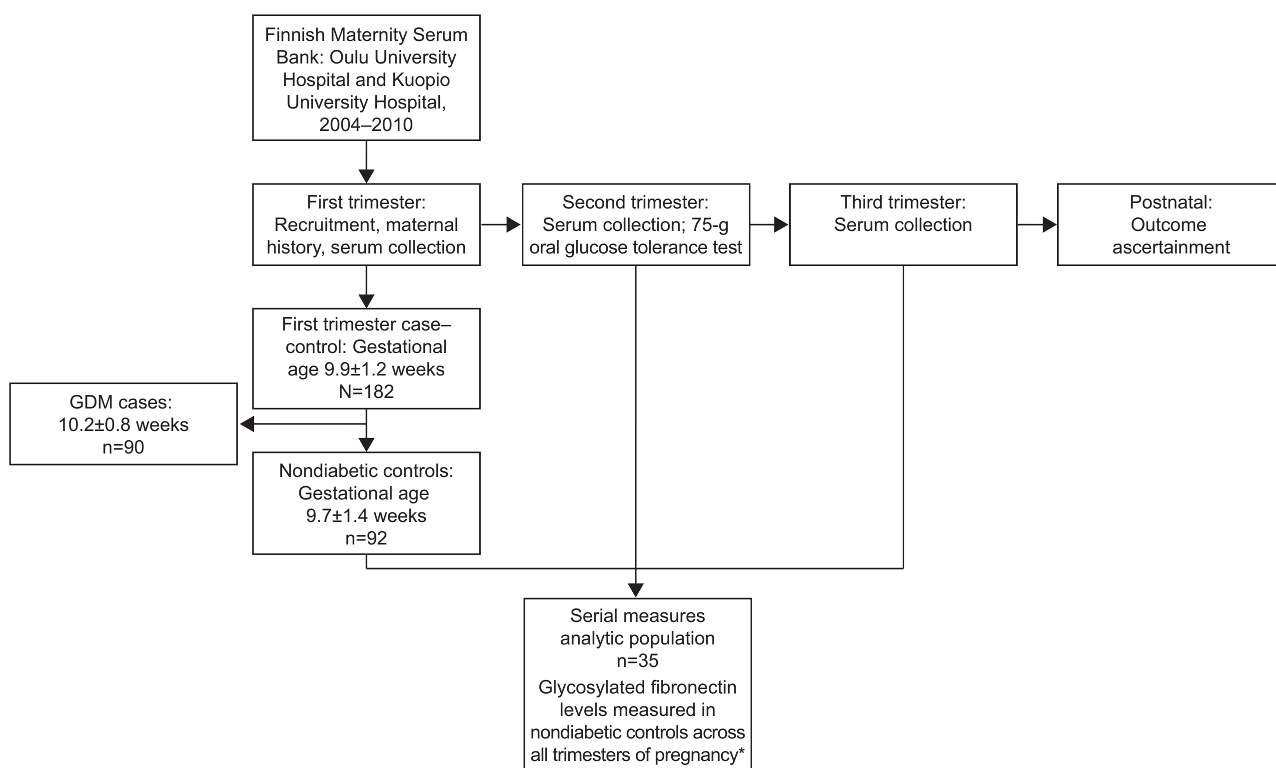

Fig. 1. Timeline chart and derivation of the first-trimester case-control and glycosylated fibronectin serial measures analytic populations. *Gestational age during the first trimester was 9.8±1.3 weeks; during the second trimester, gestational age was 24.1±1.8 weeks; during the third trimester, gestational age was 35.6±1.8 weeks. GDM, gestational diabetes mellitus.
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panel for prediction of GDM, we generated receiver operating characteristic (ROC) curves using predicted probabilities from logistic regression modeling. The area under the curve (AUC) and 95% CI were computed from simple logistic regression to describe the GDM classification ability of each serum analyte individually. A multianalyte GDM screening panel comprising glycosylated fibronectin, adiponectin, high-sensitivity CRP, and placental lactogen was assessed to determine the classification performance of the multianalyte model compared with single analytes.[17] Additional ROC curves were generated to determine weather the classification performance of the multianalyte panel varied by maternal parity, history of GDM, or time to GDM diagnosis.[17] Measures of sensitivity and specificity were reported for each analyte demonstrating a significant association with GDM status. Thresholds were chosen based on clinical utility (high-sensitivity CRP) or when a specificity of 90% was achieved. Corresponding positive predictive values and negative predictive values were computed for glycosylated fibronectin using the same threshold (120 mg/L). Low and high prevalence estimates (6% and 18%, respectively) were used for calculation of hypothetical population predictive values in addition to the actual population prevalence of 12%. The 95% CI for each predictive value was obtained using logit transformation to account for proportions near the boundary of 0 and 1.[18]

Two-sided *P* values are reported, and $P<.05$ was considered statistically significant. All statistical analyses were performed using SAS software version 9.3 and Stata software version 10.1.

RESULTS

The mean gestational age at sample collection was 9.9 (standard deviation [SD] 1.2) weeks (range 5.7–13.1), with a modest statistically significant difference between GDM and nondiabetic control participants (mean 9.7 [SD 1.4] weeks and 10.1 [SD 0.8] weeks, respectively; $P=.009$). The GDM participants were older and less likely to be nulliparous than control group participants (Table 1). In GDM participants, the mean gestational age at diagnosis was 22.2 (SD 6.2) weeks, with an average of 12.6 (SD 6.0) weeks between the first-trimester serum sample and GDM diagnosis. Of these, 10 (11%) women had a history of GDM and five (6%) required insulin therapy.

First-trimester concentrations of glycosylated fibronectin, adiponectin, high-sensitivity CRP, and placental lactogen were significantly associated with GDM status ($P<.001$; Table 1). In particular, glycosylated fibronectin demonstrated markedly higher concentrations in GDM compared with control group participants, with minimal overlap in group distributions (mean 132 [95% CI 124–139] mg/L compared with 80 [95% CI 72–87] mg/L, respectively; $P<.001$). The GDM status was not associated with first-trimester sex hormone–binding globulin concentration. In multivariate analyses, glycosylated fibronectin and adiponectin remained independently associated with GDM after adjustment for maternal factors alone and after including other serum biomarkers in the model (Table 2). In addition, there was minimal change in the ORs of glycosylated fibronectin and adiponectin on inclusion of other serum analytes. In contrast, high-sensitivity CRP demonstrated marked attenuation in the OR on addition of placental lactogen and was not associated with GDM status in the fully adjusted model.

Because statistically significant associations with a disease or outcome do not always translate into strong diagnostic accuracy,[19] we quantified the clinical utility of these tests using ROC analyses. The ROC curve representing the performance of glycosylated fibronectin alone, as illustrated in Figure 2 and Table 3, corresponded to an AUC of 0.91 (95% CI 0.87–0.96). Despite strong univariate associations with GDM status, high-sensitivity CRP, adiponectin, and placental lactogen demonstrated only marginal classification performance. As shown in Figure 2, a multianalyte model including glycosylated fibronectin, adiponectin, high-sensitivity CRP, and placental lactogen yielded an AUC of 0.92 (95% CI 0.88–0.96), which was not significantly different than that of the performance of glycosylated fibronectin alone ($P=.481$). There was no significant difference between adiponectin, high-sensitivity CRP, or placental lactogen with regard to AUC values.

On categorization at 107 mg/L, glycosylated fibronectin demonstrated a sensitivity of 81% (95% CI 73–89%) and specificity of 90% (95% CI 84–96%). Adiponectin demonstrated the next best performance, but it only yielded a sensitivity of 28% (95% CI 19–37%) at the same level of specificity (Table 3). Using a threshold of 120 mg/L, glycosylated fibronectin correctly identified 57 out of 90 individuals with GDM within the study population. At the population prevalence of 12%, this translates into positive predictive value and negative predictive value of 63% (95%% CI 53–72%) and 95% (95% CI 94–95%), respectively (Table 4). On applying a hypothetical prevalence of 18% to mimic values based on adoption of the International Association of Diabetes and Pregnancy Study Groups criteria,[20] the positive predictive value and negative predictive value for glycosylated fibronectin at the same threshold were 73% (95% CI 65–81%) and 92%

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Table 1. Maternal Clinical Characteristics and First-Trimester Serum Biomarker Concentrations by Gestational Diabetes Mellitus Status

Clinical Characteristics and Biomarker Concentrations	Nondiabetic Controls (n=92)	GDM (n=90)	P*
Maternal age (y)	26.2±4.0	31.3±6.0	<.001
Gestational age at sample collection (wk)	9.7±1.4	10.1±0.8	.009
Gestational age at delivery (wk)†	40.2±1.1	39.7±1.6	.03
Neonatal birth weight (g)†	3,563±401	3,644±512	.26
Nulliparity†	68 (82)	29 (32)	<.001
Macrosomia (more than 4,000 g)	12 (16)	20 (22)	.36
Glycosylated fibronectin (mg/L)	80±4.0	132±36	<.001
Adiponectin (mg/mL)	3.0±1.2	2.5±0.9	.001
SHBG (nmol/L)	91±66	84±46	.43
High-sensitivity CRP (mg/L)	0.39 (0.17–1.24)	1.17 (0.52–2.04)	<.001
Placental lactogen (ng/mL)	0.22 (0.05–0.36)	0.34 (0.23–0.63)	<.001

GDM, gestational diabetes mellitus; SHBG, sex hormone–binding globulin; CRP, C-reactive protein.
Data are mean±standard deviation or n (%) unless otherwise specified.
* Group differences were evaluated using parametric and nonparametric *t* tests for continuous variables and χ^2 tests for categorical variables.
† Gestational age at delivery, birth weight, and parity data were unavailable for 20, 19, and 16 nondiabetic control group participants, respectively.

(95% CI 92–93%) (Table 4). Stratification by maternal characteristics was performed to determine whether they influenced the classification performance of glycosylated fibronectin. When GDM participants with a history of GDM were excluded from the analyses (n=10), the AUC of glycosylated fibronectin remained the same (0.92; 95% CI 0.87–0.97). To determine whether there was any effect of the variation in the gestational age at which the OGTT was performed in GDM case group participants, we performed an analysis restricted to case group participants with a GDM diagnosis occurring more than 10 weeks since first-trimester sample collection. In this group, the glycosylated fibronectin AUC was similar to that seen in the entire study population (0.92; 95% CI 0.87–0.96), indicating that the time between first-trimester screening and diagnosis of GDM does not influence the classification performance of glycosylated fibronectin. Furthermore, we observed no correlation between glycosylated fibronectin concentration and time between first-trimester sample collection and GDM diagnosis ($r=-0.06$). Body mass index (calculated as weight (kg)/[height (m)]^2) values were available only for the GDM group and were not correlated with glycosylated fibronectin levels (r=0.1; data not shown).

In the 35 nondiabetic control participants with serial serum measures, glycosylated fibronectin concentration varied across the first, second, and third trimesters (mean 86 [95% CI 78–94] mg/L, 70 [95% CI 62–78] mg/L, and 82 [95% CI 74–90] mg/L, respectively; $P<.001$). There was no significant difference between the first and third trimesters with

Table 2. Unadjusted and Adjusted Odds Ratios of Gestational Diabetes Mellitus Development for Selected First-Trimester Serum Biomarkers

			Adjusted Models			
	Unadjusted Models		Maternal Factors*		Maternal Factors and Serum Glycoproteins†	
Biomarker	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Glycosylated fibronectin (mg/L)	1.06 (1.04–1.07)	<.001	1.05 (1.03–1.07)	<.001	1.05 (1.03–1.07)	<.001
Adiponectin (mg/mL)	0.62 (0.46–0.84)	.002	0.62 (0.43–0.90)	.013	0.60 (0.37–0.98)	.040
High-sensitivity CRP (mg/L)	1.64 (1.28–2.10)	<.001	1.52 (1.10–2.09)	.011	1.25 (0.84–1.86)	.268
Placental lactogen (ng/mL)	1.49 (1.20–1.85)	<.001	1.46 (1.11–1.94)	.008	1.73 (1.17–2.56)	.007

OR, odds ratio; CI, confidence interval; CRP, C-reactive protein.
* Adjusted for maternal age, gestational age at sample collection, and nulliparity.
† Adjusted for maternal age, gestational age at sample collection, nulliparity, glycosylated fibronectin, adiponectin, high-sensitivity C-reactive protein, and placental lactogen.

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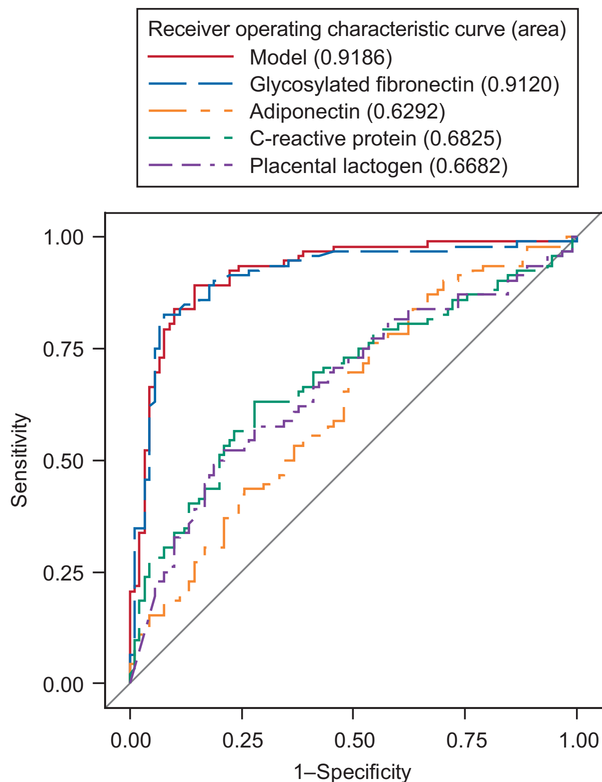

Fig. 2. Receiver operating characteristic curves illustrating the classification performance of each serum biomarker individually and as a multianalyte model. The area under the curve is presented for each corresponding curve.

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regard to glycosylated fibronectin concentration (*P*=.506). Despite this variation, nondiabetic control participants had glycosylated fibronectin distributions well below the 25th percentile of the first-trimester GDM population (Fig. 3).

DISCUSSION

Gestational diabetes increases the risk of a number of maternal-fetal disorders, including macrosomia, shoulder dystocia or other birth injuries, premature delivery, and preeclampsia.[21] In addition, 5–10% of women with GDM are found to have diabetes immediately after pregnancy, and women who have had GDM have a 34% risk of development of diabetes within the next 2–11 years compared with a risk of 5% for women without GDM in previous pregnancies.[22] Children of mothers with GDM have a 21% risk of development of type 2 diabetes in later life compared with a risk of 4% for children of mothers without GDM.[23]

The incidence of GDM is increasing, paralleling the overall increase in obesity and type 2 diabetes.[24] Because 80–90% of GDM can be managed with nutritional consultation alone,[1] universal screening for GDM is increasingly considered justified.[25] The demonstration that effective treatment of GDM reduces obstetric morbidity[26–28] provides additional support for widespread screening early in pregnancy. A recent analysis suggests that interventions to prevent the onset of GDM also can result in significant savings in health care costs.[29]

This assessment of first-trimester maternal serum glycoproteins demonstrated that the glycosylated fibronectin concentration could be an early predictor of GDM. The association between glycosylated fibronectin and GDM was independent of maternal age, parity, gestational age at sample collection, and other biomarkers such as adiponectin, high-sensitivity CRP, and placental lactogen. The classification performance of glycosylated fibronectin alone, in terms of specificity and sensitivity, suggests its potential utility as a first-trimester screening test for GDM, with performance that may surpass that of the 50-g, 1-hour OGTT, and it was not affected by time to diagnosis

Table 3. Gestational Diabetes Mellitus Classification Performance of Individual Biomarkers and Multianalyte Model

			Individual Biomarker Classification Performance (95% CI)		
Biomarker	**AUC (95% CI)**	***P***	**Threshold**	**Sensitivity (%)**	**Specificity (%)**
Glycosylated fibronectin	0.91 (0.87–0.96)	Referent	107 mg/L	81 (73–89)	90 (84–96)
High-sensitivity CRP	0.68 (0.60–0.76)	<.001	3.0 mg/L	16 (8–23)	91 (86–97)
Placental lactogen	0.67 (0.59–0.75)	<.001	0.80 ng/mL	14 (7–22)	90 (84–96)
Adiponectin	0.63 (0.55–0.71)	<.001	1.75 micrograms/mL	28 (19–37)	90 (84–96)
Multianalyte model*	0.92 (0.88–0.96)	.481	—	—	—

AUC, area under the curve; CI, confidence interval; CRP, C-reactive protein.
*The multianalyte model for receiver operating characteristic curve analysis includes glycosylated fibronectin, high-sensitivity CRP, placental lactogen, and adiponectin.

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Table 4. Glycosylated Fibronectin Gestational Diabetes Mellitus Predictive Values at Varying Prevalence Estimates

Predictive Value	Threshold (mg/L)	Predictive Value (95% CI) With Varying Prevalence Estimates*		
		6%	12%	18%
Positive predictive value	120	45 (35–55)	63 (53–72)	73 (65–81)
Negative predictive value	120	98 (97–98)	95 (94–95)	92 (92–93)
Positive predictive value	107	34 (28–41)	52 (45–59)	64 (57–70)
Negative predictive value	107	99 (99–99)	97 (97–98)	96 (95–96)

CI, confidence interval.
* Gestational diabetes mellitus prevalence within the source population is 12%.

or history of GDM. The addition of other proposed GDM biomarkers in a multianalyte model did not improve the ability to detect GDM compared with glycosylated fibronectin alone. The performance of glycosylated fibronectin in this study exceeded that of other recently evaluated first-trimester parameters, including maternal serum tissue plasminogen activator and high-density lipoprotein,[30] maternal serum visfatin,[31] OGTT-derived insulin sensitivity indices,[32] or soluble (pro)renin receptor.[33]

Like fibronectin, high-sensitivity CRP and adiponectin are also glycoproteins. The glycosylation of high-sensitivity CRP is altered in pathologic conditions,[34] whereas the glycosylation of adiponectin modulates its serum stability.[35] Their association with GDM in our study is consistent with the previous literature[6–16] and further supports the notion that the glycoproteome constitutes an important class of biomarkers that is intimately connected to cellular function and pathophysiology. However, despite their statistically significant associations, the degree of separation between the distributions of case group participants and control group participants for these previously described analytes was not adequate for their use as a screening test, as illustrated by their relatively modest AUC values. One finding of our study that is not consistent with the previous literature[7,10–12] was the lack of association observed for sex hormone–binding globulin.

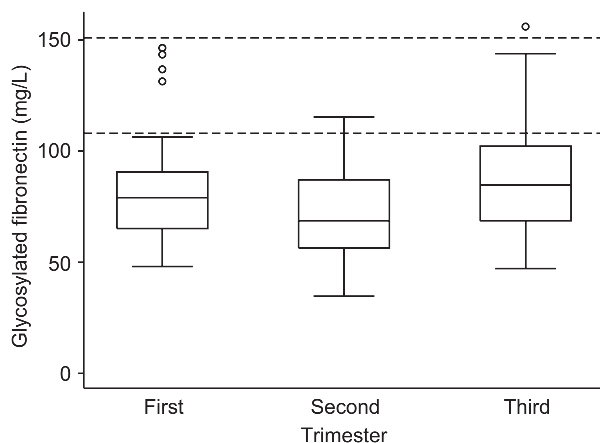

Fig. 3. Box plot of glycosylated fibronectin concentration across trimesters in 35 nondiabetic control group participants. The dashed lines represent the 25th and 75th percentiles of the gestational diabetes mellitus study group.

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The serial-measures analysis tested the variation in glycosylated fibronectin concentration across trimesters in a subset of nondiabetic control group participants. Within this analysis, we observed a statistically significant difference in second-trimester glycosylated fibronectin concentration compared with levels during the first and third trimesters. It is possible this finding reflects gestational changes in maternal glucose metabolism and may add further support for the sensitivity of glycosylated fibronectin to detect these changes. Despite the variation observed across trimesters, the absolute level of glycosylated fibronectin in this sample of nondiabetic participants was generally well below the range observed in first-trimester GDM samples.

Case-control designs may overestimate the measure of association because of the nature of the design and cumulative sampling. However, because the case group participants and control group participants were obtained from the same source population within this study, and because the prevalence of GDM was relatively low (12%), the ORs presented are likely a good estimate of the true relative risk. Another potential source of bias is the variation in time between sample collection and administration of the OGTT in GDM case group participants. However, when the analysis was restricted to participants with a minimum of 10 weeks between their first-trimester serum sample and GDM diagnosis, the results remained the same, suggesting that glycosylated fibronectin classification performance did not simply reflect GDM case group

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participants with imminent disease. Furthermore, there was no correlation between glycosylated fibronectin concentration and time to diagnosis. Additional studies comparing first-trimester glycosylated fibronectin levels with changes in maternal body mass index, pregnancy hypertension, and postpartum diabetes will provide more insight into the role of glycosylated fibronectin in GDM.

In summary, our data suggest that glycosylated fibronectin represents a promising first-trimester screening test for identifying women at risk for development of GDM. Further validation of the glycosylated fibronectin test in prospective studies will strengthen its potential as an alternative to the OGTT.

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